

Technical note

DNase treatment following thawing of Cryopreserved PBMC is a procedure suitable for lymphocyte functional studies[☆]

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Abstract

Testing freshly isolated PBMC is not practical for immune monitoring analysis in large clinical trials or natural history studies. Thus, cryopreserved PBMC represent a more practical alternative. However, cell clumping is a common problem following thawing of PBMC isolated from blood that was previously transported and stored. Cell clumping leads to loss of cells, and could affect cell function and/or phenotype. The development and validation of procedures that prevent cell clumping and preserve cell function and surface marker expression levels are necessary to allow evaluation of immune function and phenotype in cryopreserved samples from clinical studies.

The incorporation of a DNase treatment step in the standard thawing procedure efficiently avoided clump formation, and did not result in detectable changes in cell viability, expression of standard leukocyte surface markers or two key parameters of immune function, proliferation and cytokine induction in response to a variety of common stimuli. Therefore, this procedure seems suitable for standard immunologic assays.

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1. Introduction

The preparation of peripheral blood mononuclear cells (PBMCs) from whole blood is a widely used standard protocol in clinical immunology laboratories. Preparations of PBMCs can be frozen and stored at low temperatures, which allows for long-term storage and transportation. This is particularly advantageous for some studies that are conducted in settings where adequate infrastructure for sample analysis is not easily accessible. Numerous studies have used cryopreserved PBMCs in cellular immunity studies (Smith et al., 2001; Disis et al., 2005). However, cell clumping following thawing is a common problem when

Abbreviations: DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; Flu, Influenza virus; GM-CSF, granulocyte–monocyte colony stimulating factor; IL, interleukin; IFN, interferon; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; RNase, ribonuclease; TNF, tumor necrosis factor.

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working with cryopreserved cells. This problem is even more pronounced when blood is transported and stored overnight before isolation of PBMC. Cell clumping can be partly explained by the fact that granulocytes are short-lived cells. Following overnight storage, granulocytes would be no longer viable and would release DNA that could cause cell clumping. Since formation of aggregates during thawing leads to loss of cells and may compromise cell function, the development and validation of methods to prevent clumping of cells after thawing while retaining T cell function will greatly facilitate incorporation of immune monitoring in clinical studies with available cryopreserved PBMC. Previous reports have successfully used a treatment with recombinant human deoxyribonuclease (DNase) in the cell-thawing step in order to avoid clot formation in preparations of umbilical cord blood cells (Smith et al., 2001; Eichler et al., 2002; Beck et al., 2003).

In this study, we assessed the effect of DNase treatment of PBMC on standard immunophenotyping (Pinto et al., 2005) and lymphocyte functional assays that are routinely used in immune monitoring studies of patients with a variety of conditions. We confirmed that this treatment is effective for avoiding aggregate formation and improving overall recovery. DNase treatment of PBMC did not result in any significant changes in the expression of several leukocyte surface markers and lymphocyte function as measured by proliferation or cytokine induction in response to mitogens and antigens.

2. Materials and methods

2.1. Thawing/ DNase treatment

Blood samples were obtained from 24 healthy volunteers at the Occupational Health Services at NCI-Frederick (Frederick, MD), and PBMCs from freshly isolated blood samples ($n=19$) or 24 h old samples ($n=5$) were prepared as previously described (Pinto et al., 2005). Cryopreserved PBMCs were quickly thawed at 37 °C, washed once with RPMI-1640 media (Invitrogen, Grand Island, NY) supplemented with 20% heat inactivated Fetal Calf Serum (FCS, Hyclone, Logan, UT), 2 mM L-glutamine (Gibco-Invitrogen, Grand Island, NY), 100 µg/mL Penicillin-100 U/mL streptomycin (Sigma, St. Louis, MO), and 10 mM HEPES (Sigma), and resuspended in 10 mL RPMI-1640 media containing 10% FCS. The cell suspension was split into two aliquots, and placed in 50 mL conical tubes. 150 units of RNase free DNase I (15 µL at 10 units/ µL, Roche Diagnostics, Mannheim, Germany) was added to one aliquot and mixed briefly. Both samples were incubated at 37 °C, 6% CO₂ for 1 h. After incubation, samples were visually

inspected to determine the presence of cell clumps. Cells were then washed in 50 mL 10% FCS RPMI, and resuspended in 2 mL at a concentration of 2×10^6 cells/mL in AIM-V media (Gibco-Invitrogen). An aliquot was taken, washed by centrifugation and resuspended in FACS Wash buffer (PBS (Gibco)+0.7% BSA (Sigma) and 0.09% NaN₃ (Sigma)) for phenotypic analysis using flow cytometry (Pinto et al., 2005).

2.2. Proliferation / cytokine induction assays

200,000 cells/well were plated in triplicate for each condition in 96 well round bottom plates (Corning Inc, Corning, NY). Cryopreserved PBMCs isolated from freshly collected blood from 10 donors were cultured in one of the following conditions: AIM-V media as a negative control, 2 µg/mL phytohemagglutinin (PHA, 1/100, Sigma), tetanus toxoid (10 µg/mL, Aventis Pasteur, Lyon, France), or Influenza A virus (Flu, H3N2, 1/100 ATCC, Manassas, VA). Cells were incubated at 37 °C, 6% CO₂ for 48 h (3-day media and PHA) or 96 h (5-day media, tetanus, Flu), after which 50 µL of supernatant was removed from each well. Supernatants from each set of triplicate wells were pooled, frozen and stored at –80 °C. Cultures were pulsed with 1 µCi of [³H]-Thymidine (Amersham Biosciences, Buckinghamshire, UK) for 18 h before harvesting and counting in an automated scintillation counter (Microbeta, Perkin-Elmer, Boston, MA). Results were expressed as mean counts per minute (cpm) or as a Stimulation Index (SI = cpm of cultures in the presence of mitogen or antigen/cpm in the presence of media).

2.3. Flow cytometry

Flow Cytometric analysis was performed on samples from 14 donors using an FC500 Flow Cytometer (Beckman-Coulter, Fullerton, CA) for a panel consisting of anti-CD3, CD4, CD8, CD45, CD19, CD16/56 and CD14 monoclonal antibodies, or the corresponding isotype controls (Becton Dickinson, San Jose, CA; Beckman-Coulter). 200,000 cells were stained for 15 min at room temperature in the dark using the indicated antibodies. Antibodies were used as recommended by the manufacturers. Cells were then incubated in 1 mL of lysing solution (154 mM Ammonium chloride; 10 mM potassium bicarbonate; 0.12 mM EDTA; pH 7.4), to lyse contaminating erythrocytes. After 5 min, cells were washed twice in fluorescence-activated cell sorting (FACS) buffer (0.7% bovine serum albumin and 0.01% sodium azide in PBS) and then analyzed within 4 h in a flow cytometer (FC-500, Beckman-Coulter).

2.4. Cytokine determinations

Supernatants from the cytokine induction assay were thawed and tested for 13 cytokines (IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IFN- γ , TNF- α and GM-CSF) using commercially available kits (Linco Research, Inc, St. Charles, MO) for the Luminex Multi-Cytokine Profiling Technology, following manufacturer's instructions. All specimens were tested in duplicate wells. Results are reported as the mean of the replicates. Data analysis was performed using Bioplex manager software version 2.0 (Bioplex, Bio-Rad, Hercules, CA). A five-parameter logistic curve fit was applied to each standard curve and sample concentrations were interpolated from the standard curve. The lower levels of detection for all the cytokines were 1.6 pg/ml. In this analysis, specimens with individual cytokine levels below the lower detection limit were arbitrarily assigned a value of one-half of this limit (0.8 pg/ml) for that cytokine.

2.5. Statistical analysis

The nonparametric Wilcoxon matched pairs test was used to determine statistical differences between untreated or DNase treated samples for each of the conditions tested. A two-sided- p value of 0.05 or lower was considered significant.

3. Results and discussion

To test whether DNase treatment of PBMCs could affect immune phenotype and function *in vitro*, we performed immune monitoring tests in parallel using cryopreserved cells that were thawed and then either treated with DNase or incubated with control media in the absence of DNase (untreated cells).

Average cell viability for DNase treated cells was $95 \pm 5\%$ ($n=24$), and $94 \pm 6\%$ for control cells ($n=24$), as determined using Trypan Blue exclusion (Sigma), and

average cell recovery in DNase treated and untreated specimens was $56 \pm 15\%$ and $51 \pm 19\%$, respectively. Cell aggregate formation was only observed in those samples that were not incubated with DNase. Small cell aggregates were found in 7/19 DNase-untreated PBMC specimens prepared from fresh blood. In contrast, all samples from 24-hour-old samples presented clump formation. Overall, number and size of clumps in fresh samples were smaller than in old samples (data not shown). Furthermore, when cell recoveries of specimens from fresh or 24-hour-old PBMC in the absence of DNase treatment were compared, a higher recovery was observed for PBMC from fresh blood ($59 \pm 11\%$ vs. $20 \pm 12\%$ for 24-hour-old samples). This result is in agreement with previous reports (Smith et al., 2001).

Results from immune phenotype measurements using flow cytometry are shown in Table 1. DNase treatment did not affect surface markers for T (total CD3 or CD4 and CD8 T cell subsets) B, NK cells and monocytes ($p > 0.11$). Our observation that incubation with DNase does not significantly affect the values obtained for immune phenotype markers agrees with a previous report, where no significant variation in the percentage of LFA-1, ICAM-1 and L-selectin was observed after DNase treatment (Beck et al., 2003).

However, different scatter plots (side-scatter vs. forward scatter) were observed when DNase treated cells were compared to untreated samples. A cell population of high side-scatter and low forward scatter could be observed only in cell preparations that were treated with DNase (data not shown). A likely explanation for this cell population with high side-scatter is that these cells are presumably granulocytes that shrunk upon freezing and thawing, and potentially release the DNA that could cause cell aggregation (Brinkmann et al., 2004). This view is supported by the fact that this population is not observed in DNase-untreated preparations, where these cells were probably lost upon clump formation.

Table 1
Effect of DNase treatment on PBMC phenotype marker percentage^a

Parameter	No DNase		DNase		p^b
	Median (%)	Mean (%) \pm SD	Median (%)	Mean (%) \pm SD	
T cells	74.5	75.6 \pm 7.2	74.9	75.6 \pm 7.2	0.90
CD3 ⁺ CD4 ⁺ Cells	48.1	49.4 \pm 8.0	46.9	48.9 \pm 7.3	0.58
CD3 ⁺ CD8 ⁺ cells	20.3	24.4 \pm 11.6	22.4	24.7 \pm 11.1	0.43
B cells	12.2	14.3 \pm 9.2	11.5	13.9 \pm 8.8	0.19
NK Cells	8.4	8.2 \pm 3.9	8.4	8.5 \pm 3.7	0.12
Monocytes	20.8	19.7 \pm 7.1	17.4	18.5 \pm 7.0	0.24

^a $n = 14$ donors.

^b p values were calculated using Wilcoxon matched pairs test. $p < 0.05$ was considered significant.

Table 2

Effect of DNase treatment on lymphocyte proliferation^{a,b}

Stimulus	No DNase		DNase		<i>p</i> ^c
	Median	Mean ± SD	Median	Mean ± SD	
Media–3 days	1119	1533 ± 1148	1057	1352 ± 1021	0.04
PHA	212,566	214,623 ± 43,388	213,841	210,930 ± 38,246	0.49
Media–5 days	2358	2759 ± 1511	1734	2561 ± 2167	0.49
Tetanus	23,192	27,045 ± 15,686	19,548	24,054 ± 14,544	0.32
Flu	24,962	27,828 ± 15,866	21,051	26,251 ± 15,144	0.43

^a *n* = 10 donors.^b results are expressed in counts per minute.^c *p* values were calculated using Wilcoxon matched pairs test. *p* < 0.05 was considered significant.

Next, we evaluated whether DNase treatment influences the results obtained with immunologic functional assays. *In vitro* proliferative responses and cytokine profiling are two key assays often used as a measurement of cell mediated immunity. DNase treated or untreated cells were stimulated with recall antigens (tetanus or influenza virus), a mitogen (PHA) or incubated in the presence of media alone as a negative control. Supernatants from these cultures were collected for cytokine profiling analysis using a multiplex detection system. Immune response results in DNase treated and untreated samples are shown in Tables 2 and 3. DNase treatment did not affect proliferative responses to any of the stimuli used. In general, proliferation values obtained from samples treated with DNase are slightly lower than for

untreated samples, but the magnitude of these differences is too small to be considered relevant for this biological system. Proliferation of unstimulated cells after 3 days of culture was slightly but statistically significantly reduced in DNase treated samples. Median proliferation observed for DNase-untreated (1119 cpm) cells was only 5.9% higher than that of treated (1057 cpm) cells. Moreover, the results for unstimulated cells used as control for antigenic responses (5 days of incubation) did not show a significant change (Table 2). In addition, no pronounced differences were observed in a small (*n* = 4) number of samples for any of the measured cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IFN-γ, GM-CSF and TNF-α) when DNase treated and untreated samples were compared. Although

Table 3

Effect of DNase treatment on cytokine production^{a, b, c}

Parameter	PHA		Tetanus toxoid		Flu	
	No DNase	DNase	No DNase	DNase	No DNase	DNase
	Mean ± SD (median)	Mean ± SD (median)	Mean ± SD (median)	Mean ± SD (median)	Mean ± SD (median)	Mean ± SD (median)
IL-1β	39 ± 14 (42)	51 ± 12 (52)	10 ± 7 (8)	12 ± 8 (10)	3 ± 4 (ND ^d)	ND
IL-2	5041 ± 3451 (3560)	4901 ± 3047 (3839)	45 ± 30 (44)	48 ± 35 (52)	45 ± 35 (39)	57 ± 34 (58)
IL-4	1422 ± 650 (1329)	1399 ± 488 (1425)	22 ± 21 (18)	27 ± 35 (14)	22 ± 15 (19)	30 ± 17 (31)
IL-5	21 ± 12 (21)	22 ± 12 (20)	3 ± 2 (3)	4 ± 3 (2)	ND	ND
IL-6	6388 ± 3111 (7401)	8042 ± 1926 (8317)	2945 ± 3553 (1296)	3473 ± 3191 (2207)	126 ± 218 (22)	25 ± 6 (25)
IL-7	22 ± 1 (22)	22 ± 1 (22)	5 ± 3 (5)	6 ± 4 (6)	3 ± 1 (3)	2 ± 1 (ND)
IL-8	9988 ± 23 (10,000)	10,000 ± 0 (10,000)	10,000 ± 0 (10,000)	9852 ± 296 (10,000)	5301 ± 4554 (5040)	4997 ± 2571 (5104)
IL-10	1007 ± 628 (932)	1010 ± 463 (1088)	23 ± 17 (18)	30 ± 16 (29)	54 ± 35 (55)	62 ± 32 (60)
IL-12	126 ± 80 (126)	150 ± 83 (162)	2 ± 1 (ND)	2 ± 1 (ND)	ND	ND
IL-13	2464 ± 680 (2669)	2583 ± 490 (2692)	222 ± 219 (193)	240 ± 208 (265)	61 ± 54 (36)	78 ± 50 (62)
IFN-γ	6661 ± 2492 (6283)	7300 ± 1383 (6946)	618 ± 584 (566)	646 ± 647 (567)	290 ± 199 (206)	296 ± 152 (231)
GM-CSF	1265 ± 580 (992)	1324 ± 486 (1128)	113 ± 95 (115)	118 ± 97 (130)	36 ± 18 (30)	40 ± 15 (36)
TNF-α	2000 ± 0 (2000)	2000 ± 0 (2000)	197 ± 143 (179)	227 ± 154 (209)	59 ± 23 (59)	52 ± 16 (49)

^a Results are expressed as median or mean pg/mL from 4 different donors.^b Only IL-6, IL-8 and TNF-α were present at detectable levels in media controls:

3-day media (no DNase/DNase): IL-6 (6/5); IL-8 (2011/2720); TNF-α (12/11)

5-day media (no DNase/DNase): IL-6 (5/7); IL-8 (2697/2337); TNF-α (8/10)

^c All *p* values were non-significant (*p* ≥ 0.12, calculated using Wilcoxon matched pairs test).^d ND: not detected (< 1.6 pg/ml).

a trend towards higher values for samples from DNase treated cells can be observed, these differences are very small to be functionally relevant.

In conclusion, treatment of PBMCs with DNase after thawing is an appropriate procedure to avoid the formation of cell aggregates, and is therefore of benefit when working with samples where cell clumping could decrease cell recovery and content. This procedure does not appear to alter the results obtained in phenotype marker expression measurements, lymphocyte proliferation or cytokine induction analyses. Analysis of functional differences between freshly isolated and 24-hour-old PBMC samples was beyond the scope of the present study.

Although the treatment of PBMC with DNase should be validated for further methods, the results presented in this report suggest that incorporation of a DNase incubation step in a standard thawing procedure is suitable for immune functional analysis of cryopreserved samples in which cell clump formation is an issue. This will allow for immune monitoring studies using cryopreserved samples from overnight stored blood.

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References

- Beck, C., Nguyen, X.D., Kluter, H., Eichler, H., 2003. Effect of recombinant human deoxyribonuclease on the expression of cell adhesion molecules of thawed and processed cord blood hematopoietic progenitors. *Eur. J. Haematol.* 70, 136.
- Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., Zychlinsky, A., 2004. Neutrophil extracellular traps kill bacteria. *Science* 303, 1532.
- Disis, M.L., Dela Rosa, C., Goodell, V., Kuan, L.Y., Chang, J.C., Kuus-Reichel, K., Clay, T.M., Kim Lyerly, H., Bhatia, S., Ghanekar, S.A., Maino, V.C., Maecker, H.T., 2005. Maximizing the retention of antigen specific lymphocyte function after cryopreservation. *J. Immunol. Methods*.
- Eichler, H., Beck, C., Bernard, F., Bugert, P., Kluter, H., 2002. Use of recombinant human deoxyribonuclease (DNase) for processing of a thawed umbilical cord blood transplant in a patient with relapsed acute lymphoblastic leukemia. *Ann. Hematol.* 81, 170.
- Pinto, L.A., Trivett, M.T., Wallace, D., Higgins, J., Baseler, M., Terabe, M., Belyakov, I.M., Berzofsky, J.A., Hildesheim, A., 2005. Fixation and cryopreservation of whole blood and isolated mononuclear cells: influence of different procedures on lymphocyte subset analysis by flow cytometry. *Cytom. B Clin. Cytom.* 63, 47.
- Smith, J.G., Liu, X., Kaufhold, R.M., Clair, J., Caulfield, M.J., 2001. Development and validation of a gamma interferon ELISPOT assay for quantitation of cellular immune responses to varicella-zoster virus. *Clin. Diagn. Lab. Immunol.* 8, 871.